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Acclimation and Selection for Methanesulfonic Acid (MSA) Degrading Microbial Population Using Continuous Culture Techniques

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Methanesulfonic acid (MSA) is indicated to be a missing link in the biogeochemical sulfur cycle. Until recently, the fate of MSA in the environment was largely unknown. The Environmental Engineering Laboratory (EEL) at Merck & Co., Inc. investigated the biodegradation potential and fate of MSA in a typical wastewater treatment system. This study was undertaken to methodically acclimate, select, and enrich for MSA degrading microorganisms using continuous culture (CC) techniques, carried out over several months. Complete mineralization of MSA would produce carbon dioxide and sulfate. Hence, to achieve a precise material balance, a direct Ion Chromatography method with good precision was developed for the analysis of MSA and sulfate in an activated sludge matrix.

Prolonged acclimation (at least 4 residence times for each concentration) resulted in the selection of MSA degrading microorganisms in an activated sludge system. Complete mineralization was achieved at MSA concentrations ranging from 5 to 1000 mg/L. Sulfate generation, a result of MSA biodegradation, increased with increasing MSA loading and was equal to the theoretical value of one mole sulfate per mole of MSA. Ion chromatography analyses of the effluent sulfate concentration confirms the MSA to sulfate mass balance at various MSA loadings used in this study. Total Organic Carbon (TOC), Total Oxygen Demand (TOD) and pH were measured during the course of the study. Average TOC and TOD removal were greater than 94 and 91%, respectively, for MSA concentrations up to 1000 mg/L. The pH was monitored throughout the study and was maintained in the range 6.8 to 7.8 through the addition of sodium bicarbonate. The data gathered from this study indicate that the MSA degrading organisms have a slow growth rate (specific growth rate $\leq$O .017 hr^{-1}) . The Hydraulic Residence Time (HRT) was maintained at approximately 60 hours. The optimum HRT for the microorganisms in the CC bioreactor was found to be 240 and 160 hours. HRT's below 40 hours resulted in less than 100% degradation of MSA and ultimate washout.

The activated sludge organisms were gradually challenged with increasing MSA concentrations (a factor of 12). Rapid increases in MSA concentrations were found to be inhibitory. Biodegradation

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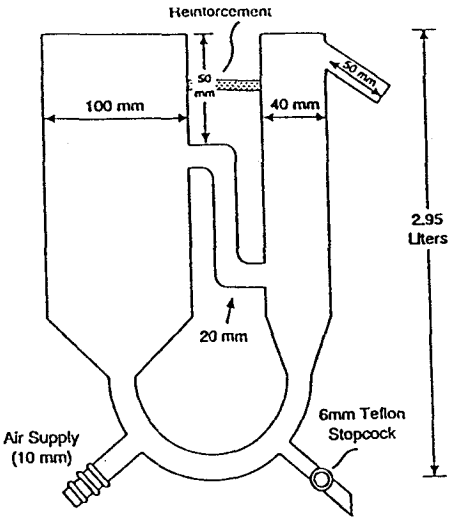

Figure 1. Three liter continuous culture bioreactor.

of 1000 mg/L MSA required several months of slow step-wise acclimation with increasing MSA concentrations. Ultimately, ~100% of the MSA was degraded at 1000 mg/L loading. Inhibition was evident at a concentration of 2000 mg/L MSA. Therefore, as long as the MSA concentration does not exceed 1000 mg/L, the sewering of MSA to a well acclimated activated sludge treatment plant should pose no acute adverse impact on it's performance. This has been tested and verified in a large-scale activated sludge treatment plant treating $2X10^6$ Liters/day. These results point to the fact that MSA degraders are probably ubiquitous in nature.

MATERIALS AND METHODS

A glass continuous culture (CC) bioreactor, designed by the authors and manufactured by Crown Glass Co., Somerville, NJ was used in the study. A schematic of the 3 Liter CC bioreactor is shown in Figure 1.

The volume of the bioreactor was 2.95 liters. The ratio of the aeration to settling volumes is ~7. The HRT of the CC bioreactor was maintained at approximately 60 hours throughout the study. Sludge loading was kept stable at approximately 6 g/L by appropriate wasting of mixed liquor suspended solids (MLSS) through the stopcock port located at the bottom of the CC bioreactor. All chemicals used were reagent or HPLC grade materials and distilled water was used for all solution preparations. The vitamin and inorganic micronutrients solution was prepared as described in OECD Guidelines, "301E Modified OECD Screening Test (1993)' except that all sulfate salts in the multi minerals solution were replaced with the corresponding chloride salts to help close the sulfur balance. The MSA bioreactor influent nutrient solution (per liter) was prepared by adding: 160 mg Bacto beef extract, 110 mg Bacto peptone, 90 mg Bacto Urea, 0.6 mL ethanol and 0.6 mL methanol to the above OECD vitamin and inorganic micronutrient solution. The MSA bioreactor feed solution's BOD/N/P ratio was 100/5.7/1.1. MSA concentrations were increased by a factor of ~2 from 5 to 2000 mg/L during the course of this study. In addition, the amount of sodium bicarbonate added to the MSA bioreactor solution for buffering was increased

from 0.75 to 1.5 g/L with increasing MSA concentrations. The pH of the bioreactor was thus maintained in the range, 6.8 to 8.0.

IC analysis of MSA, chloride, phosphate, sulfate, and nitrate was conducted using a Dionex DX300 Ion Chromatography system. The anions are separated using the HPIC-AS4A Exchange Separator Column with a 1.9 mM carbonate/l.8 mM bicarbonate buffer eluant. Detection is by conductivity with auto self regenerating eluant suppression. A five anion standard, purchased from Dionex, containing fluoride, chloride, nitrate, phosphate, and sulfate was utilized. The MSA standard solution was prepared by weighing 1000 mg of MSA into a 1 liter volumetric flask and diluting to the mark with distilled water. A standard IC working solution was prepared by adding 10 mL of the five anion standard solution from Dionex into a 100 mL volumetric flask to which exactly 1.0 mL of the 1000 mg/L MSA standard solution was added; the flask was then filled to the mark with distilled water. The following concentrations resulted: fluoride 2 mg/L, chloride 3.0 mg/L, MSA 10.0 mg/L, nitrate 10.0 mg/L, phosphate 15.0 mg/L, and sulfate 15.0 mg/L. The instrument detection limit was 0.05 mg/L with a sensitivity of 0.01 mg/L and an optimum concentration range of 1 - 30 mg/L MSA. Positive spiking experiments in the bioreactor solution matrix with MSA and sulfate resulted in percent recoveries of 99.2 and 99.5%, respectively. The IC analyses were performed on both influent and effluent samples which were first filtered through 0.45 micron nylon filters. TOC and TOD analyses were performed using an Ionics, Model 1270M TOD/TOC/TC Analyzer. Total solids were analyzed gravimetrically according to Standard Methods for the Examination of Water and Wastewater "2540 B: Total Solids Dried at 103-105°C (1992)". The pH was measured with an Orion #301 pH/ISE Meter and calibrated per the Orion Instruction Manual.

RESULTS AND DISCUSSION

Prolonged acclimation (at least 4 residence times for each concentration) resulted in the selection of MSA degrading microorganisms in an activated sludge system. Complete mineralization was achieved at MSA concentrations ranging from 5 to 1000 mg/L. Sulfate generation, a result of MSA biodegradation, increased with increasing MSA loading and was equal to the theoretical value of one mole sulfate per mole of MSA. Ion chromatography analyses of the effluent sulfate concentration confirms the MSA to sulfate mass balance at various MSA loadings used in this study. The data gathered from this study indicate that the MSA degrading organisms have a slow growth rate (specific growth rate ≤ 0.017 hr^{-1}). The activated sludge organisms were gradually challenged with increasing MSA concentrations (a factor of ~2). Rapid increases in MSA concentrations were found to be inhibitory. Biodegradation of 1000 mg/L MSA required several months of slow step-wise acclimation with increasing MSA concentrations. Ultimately, ~100% of the MSA was degraded at 1000 mg/L loading.

MSA was found to be completely mineralized to carbon dioxide and sulfate at concentrations up to 1000 mg/L. Higher MSA concentrations (2000 mg/L) were found to be inhibitory to the

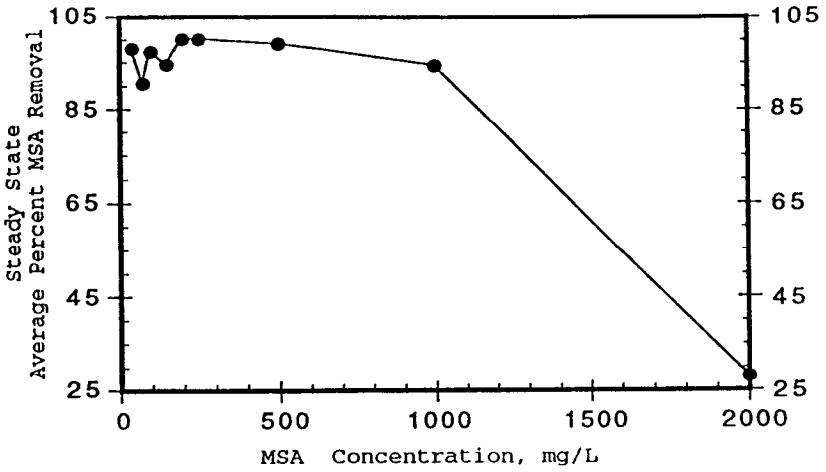

Figure 2. MSA removal as a function of MSA Concentration.

activated sludge organisms. Steady state average % MSA removal as a function of MSA concentration is shown in Figure 2. The optimum HRT, based on this study, for the microorganisms in the CC bioreactor was ≥40 and ≤60 hours. Activated sludge from a POTW treating combined domestic and industrial wastes was utilized as the inocula in this study. Nutrient media utilized in the study was tailor-made in it's composition with the omission of sulfur-containing compounds such as magnesium sulfate. This was replaced with the chloride salt of the metal compound in an effort to maintain the ionic balance of the medium. The intent was to limit the sulfur available from non-MSA sources, and create conditions which would favor those organisms capable of using MSA as a sulfur, carbon and/or energy source.

Total Organic Carbon (TOC), Total Oxygen Demand (TOD), pH, and Total Solids (TS) were evaluated during the course of this study. The influent gross parameters (in mg/L) based on a MSA influent concentration of 10 mg/L was: TOD=1447.3, TOC=578.9, N=15.4, s=3.3, and C1=298.5. The BOD/N/P ratio was 100/5.7/1.1. At 1000 mg/L MSA, the carbon and sulfur contribution from MSA to the nutrient solution was 125 and 335 mg/L, respectively. TOC and TOD removal rates averaged greater than 94 and 91%, respectively, at MSA concentrations up to 1000 mg/L. The pH was monitored throughout the study and was in the range, 6.8 to 7.8. Solids loading in the bioreactor were maintained in the range of 4.6 to 7.8 g/L throughout the study by appropriate sludge wasting.

Ion chromatography was utilized to monitor MSA biodegradation and sulfate generation in the CC bioreactor. Percent removal efficiency of MSA was greater than 99% for MSA concentrations up to 1000 mg/L MSA (based on ion chromatography assay for sulfate). Sulfate generation from the biodegradation of MSA increased with increasing MSA loading (1 mole of sulfate released/mole of MSA degraded). Ion chromatography analyses of

the effluent sulfate concentration confirms the biological oxidation of MSA (mass balance at the various MSA loadings) by the acclimated mixed microbial population. The IC analyses results for MSA and sulfate is depicted in Table 1.

Table 1. MSA biodegradation study: Ion chromatography analyses of influent and effluent for MSA and sulfate.

	Influent (mg/L)		Effluent (mg/L)		
Day	MSA	Sulfate	MSA	Sulfate	% MSA Removal
1	<0.05	2.0	<0.05	2.0	(Blank)
7	10.0	2.0	<0.05	12.2	>99.5
14	20.1	2.0	<0.05	21.0	>99.8
21	40.3	2.0	<0.05	41.0	>99.9
28	75.3	2.1	<0.05	76.1	>99.9
35	100.1	2.0	<0.05	99.9	>99.9
42	150.2	2.1	1.0	149.0	99.3
49	200.4	2.1	1.0	199.9	99.5
54	250.4	2.0	1.1	251.1	99.6
70	500.5	2.0	1.2	500.5	99.6
84	1000.1	2.1	1.0	999.9	99.9
98	2000.8	2.0	1670.1	346.1	16.5

Total Organic Carbon (TOC) and Total Oxygen Demand (TOD) parameters were successfully used as indirect measurements of the microorganisms' growth and performance during acclimation and degradation of MSA. Percent TOC and TOD removal efficiency increased as the system was well acclimated before challenging with increasing concentrations of MSA. At MSA concentration greater than 1000 mg/L (i.e., 2000 mg/L), the percent removal efficiency of both TOC and TOD declined rapidly to 16.4 and 12.9%, respectively. The TOC and TOD data are shown in Table 2.

Table 2. MSA biodegradation study: TOC and TOD analyses of influent and effluent.

	Influent (mg/L)		Effluent (mg/L)			
Day	TOC	TOD	TOC	TOD	% TOC Removal	% TOD Removal
1	578	1447	35	130	94.0	91.0
7	578	1447	35	130	94.0	91.0
14	578	1447	35	130	93.9	91.0
21	580	1450	35	130	94.0	91.0
28	586	1455	36	130	93.9	91.1
35	588	1455	35	131	94.0	91.0
42	595	1463	36	135	93.9	90.8
49	600	1496	36	140	94.0	90.6
54	605	1500	36	141	94.0	90.6
70	635	1580	38	150	94.0	90.5
84	700	1747	42	157	94.0	91.0
98	825	2065	690	1800	16.4	12.9

Baker et al (1991) demonstrated that desulfurization of aliphatic organosulfonates by biocatalytic C-S bond cleavage constitute a major pathway in the biogeochemical sulfur cycle. Frost (1991) identified that Escherichia coli K-12 bacteria can grow on MSA,

resulting in the complete mineralization of MSA to carbon dioxide and sulfate. The microorganisms involved in the biological process have been identified as facultatively heterotropic methylotrophs by Frost (1991) and Kelly et al. (1990). The literature further suggests that the ability to utilize sulfonates as a source of carbon and energy is applicable to many other microorganisms. Thysee et al. (1972) isolated two strains of Pseudomonas, AJ 1 and AJ 2, growing on the C_4-C_7 and C_8-C_{12} n-alkane-1-sulfonates, respectively, as the only source of their carbon and energy. The alkane sulfonates are dissimilated by these strains to yield carbon dioxide, water, and sulfate. More recent work by Hrsak (1995) demonstrate biodegradation of commercial linear alkylbenzenesulphonate (LAS), in the presence and absence of methane as a primary growth substrate, by type II methanotrophs isolated from a groundwater aquifer.

A wide range of sulfonates, sulfinates, and sulfones can be used by Cholerella, leading to the suggestion that the green algae could be important in the biodegradation of such compounds (especially xenobiotics) in nature. Naphthalenesulfonic and

benzene sulfonic acids are present in many wastewaters and are cited as ~10% of the pollution burden in the Rhine River by Kelly et al. (1990). These compounds are desulfonated and used as sulfur and carbon sources for growth by several bacteria and algae Specifically, sulfanilic acid 4-aminonbenzenesulfonate (4-ABS) supported the growth of a binary mixed culture consisting of a Gram-negative aerobic rod and Aarobacterium radiobacter. The compound provided all carbon, nitrogen, sulfur, and energy requirements for the mixed culture.

Atmospheric dimethyl sulfide (DMS), arising from marine algae, cyanobacteria and salt marsh plants is the principal sulfur compound entering the atmosphere from terrestrial and aquatic environments. MSA has been identified as a major photochemical oxidation product of DMS. DMS and MSA are predominantly biogenic in origin and are the main gaseous links in the biogeochemical sulfur cycle. Kelly et al. (1990) determined that MSA is a stable compound and does not undergo photochemical oxidation. It's removal from the atmosphere is by wet and dry deposition. MSA partitions to the aerosol phase, as well as to nucleating droplets and is deposited in rain and snow. Analysis of Antarctic ice cores gives evidence of its global deposition over many thousands of years. Baker et al. (1991) postulated that the sulfonic acid functional group of MSA must be available for microbial utilization. Baker et al. (1991) also demonstrated that the methylotrophs and sulfur bacteria are able to derive energy from the degradation of MSA. Recently, MSA utilizers have been shown to be present in many habitats, from marine, fresh water and terrestrial environments (J. C. Murrell et a1.(1994), University of Warwick, personal communication of unpublished results). The hypothesis that MSA degrading organisms are ubiquitous and abundant in nature is well supported by the results of this work.

This study results indicate that microorganisms naturally present in any activated sludge wastewater treatment plant can be acclimated to degrade MSA. The acclimation step has to be slow with a stepwise addition of MSA to the sludge over extended time. In effect, an "environmental niche" is provided to the naturally occurring activated sludge culture by optimizing the wastewater

treatment parameters. This results in the utilization of MSA as the sole source of carbon for growth and energy and consequent degradation of MSA even at very high concentrations. Concentrations as high as 1000 mg/L MSA were determined to be degradable, utilizing MSA as the sole source of carbon and energy. Acclimating microorganisms that are present in a typical $2x10^6$ L/day activated sludge treatment plant to utilize large amounts of MSA as a carbon and energy source has also been demonstrated as part of this study. The method described is reproducible at any scale and with any starting mixed culture of activated sludge origin as long as the acclimation procedure and operating parameters are strictly followed.

Microbial degradation of MSA is critical and is a definite missing link in the biogeochemical sulfur cycle. MSA is a very stable compound. It is non volatile (B.P. ~265 °C) and all salts of MSA are freely soluble in water. It does not undergo photolysis and hydrolysis. Also, it is resistant to decomposition and degradation even at elevated temperatures and pressures. The only removal mechanism from the environment is through biocatalyzed oxidation. Thus, microorganisms play a vital role in the biogeochemical sulfur cycle.

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